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(54) Title: HYDROXYALKYL CYCLODEXTRIN-ANTIFUNGAL POLYENE ANTIBIOTICS COMPLEXES**(57) Abstract**

The present invention involves a composition of matter comprising amphotericin B and hydroxyalkyl-gamma-cyclodextrin. The hydroxyalkyl-gamma-cyclodextrin is preferably hydroxypropyl-gamma-cyclodextrin, most preferably 2-hydroxypropyl-gamma-cyclodextrin. The cyclodextrin utilized in the practice of the present invention comprises rings of eight glucose units bound in 1-4 glucosidic linkages, each glucose unit having a hydroxyalkyl radical in ether linkage. The hydroxyalkyl radical is bound in ether linkage to carbon-5 of the glucose unit. The composition of matter of the present invention preferably comprises a cyclodextrin ring of eight glucose units bound in 1-4 glucosidic linkages, substantially each glucose unit having a 2-hydroxypropyl radical in ether linkage. The present invention also involves a method for the treatment of an animal with fungal infection. This method comprises administering to the animal a fungicidally effective amount of a composition of matter as described above comprising amphotericin B and hydroxyalkyl-gamma-cyclodextrin. The administration is preferably parenteral, particularly for disseminated fungal infection, but could be internal or topical, depending upon the particular site of fungal infection and desires of the treating physician. The disseminated fungal infection most preferably treated is a yeast infection such as a *Candida albicans*, *Candida tropicalis*, *Candida parapsiloses* or *Torulopsis glabrata* infection. The fungicidally effective amount useful for treatment of disseminated fungal infection contains between about 0.4 mg amphotericin B per kg body weight and about 4.0 mg amphotericin B per kg body weight.

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Hydroxyalkyl cyclodextrin-antifungal polyene
antibiotics complexes.

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The present invention relates to a composition of
matter comprising hydroxyalkyl cyclodextrin and
20 amphotericin B. This composition is particularly useful
for treatment of disseminated fungal infection.

Infection is the cause of death in 51% of patients
with lymphoma and 75% of patients with leukemia. Although
25 bacteria are the causative organisms of many such
infections, fungi account for 13% of the fatal infections
in patients with lymphoma and for more than 20% of
patients with leukemia. The fungus Candida albicans
causes more than 80% of these infections, and Aspergillus
30 spp. is also a frequent cause of such infections. In
addition, fungal infection is a major cause of morbidity
and mortality in patients with congenital and acquired
deficiencies of the immune system.

35 Despite the fact that several new antifungal agents
have become available, amphotericin B remains the drug of

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choice for treatment of most systemic mycoses in cancer and other immuno-compromised patients. Amphotericin B, a polyene antibiotic, is a lipophilic compound which interacts with ergosterol in fungal membranes, thus
5 creating transmembrane channels which permit the escape of may ions and metabolites that are essential to the cell's continued vitality. Unfortunately, the drug also interacts appreciably with the cholesterol found in
10 mammalian cell membranes. This interaction with the cell membrane of mammalian cells is probably the basis of the toxic effects which it exerts on the mammalian kidney, hematopoietic system and central nervous system.

Amphotericin B is insoluble in aqueous solution,
15 consequently it is supplied commercially as a combination of amphotericin B, desoxycholate and buffers, suspended in a glucose solution to form a colloidal suspension for administration to the patient. It is usually given intravenously over a period of from two to six hours.
20 Faster infusions may result in cardiotoxicity. Other toxic effects of amphotericin B may manifest themselves as real disfunction, anemia, fever and hypotension. Amphotericin B is supplied commercially under the brandname FUNGIZONE® by E. R. Squibb & Sons, Inc. The
25 side effects and contraindications of FUNGIZONE® are discussed at page 1929 et seq. of the Physicians' Desk Reference, 37th Ed. (Oradell, N.J., Medical Economics Co., 1983). (The references cited in this application are incorporated by reference herein for the reasons cited.)
30

The toxicity of amphotericin B limits the total amount of the drug which may be used in the treatment of a fungal infection. Furthermore, it is often ineffective in neutropenic and immunodeficient patients, patients who are
35 highly susceptible to fungal infections. Consequently, there is a need for a system which decreases the toxicity

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of amphotericin B to the mammalian system while simultaneously enhancing its effectiveness against the fungal infection.

5 Amphotericin B is the most potent antifungal drug available for some purposes. Its use, however, is limited by its serious toxicity. Cyclodextrin represents a novel drug carrier system that has the ability to improve drug properties such as solubility, stability, and
10 bioavailability and to reduce their side effects.

 The bioavailability of drugs from their solid-state form depends primarily on their dissolution properties: fast dissolution and the ability to form a concentrated
15 solution usually result in good absorption of a drug. The dissolution characteristic of a compound is mainly a function of its structure, but to a lesser degree can be changed by manipulation of the solid state of the drug. Various polymorphs and solvates of drugs differ in rates
20 of dissolution and in solubility; the proper choice of the crystalline state thus may aid its dissolution. A drug may also be converted from a crystalline state into an amorphous state which dissolves better, unfortunately, the amorphous state is metastable. The amorphous state may be
25 obtained and stabilized by various means. The present invention concerns the complexation of a drug with water-soluble polymers (compounds which are intrinsically amorphous themselves).

30 The formation of inclusion complexes of a drug with nontoxic agents is a type of manipulation used to improve the dissolution properties of drugs. (Frank (1975) J. Pharm. Sci., 64:1585) Cyclodextrins have been used extensively as such complexation agents. In these
35 complexes molecules of the drug are enclosed in the hydrophobic cavity of a cyclodextrin molecule or in a

channel formed by several molecules of cyclodextrin. These cyclodextrin complexes are crystalline, and the structure of many of them are known.

- 5 Alpha-, beta- and gamma-cyclodextrins are cyclic oligomers of glucose respectively containing 6, 7 or 8 glucose residues. These glucose residues are arranged in a circle with a toroidal shape in which all the primary hydroxy groups are on the narrower base and secondary
- 10 hydroxy groups are on the wider base of the toroid. There are no hydroxy groups on the inside of the circle of glucose residues and consequently the cavity of the toroid has a non-polar character. In a hydrated state the cavity of alpha-, beta- or gamma-cyclodextrin is filled with 6,
- 15 11 or 17 water molecules, respectively, and these molecules of water may be replaced with a gain of energy by molecules of a non-polar compound. The formation of such complexes of cyclodextrins with drugs is a rapidly reversible reaction and these complexes exist both in
- 20 solution and in crystalline state. The structures and thermodynamics of formation of many such complexes have been carefully studied (see e.g., Saenger (1980) Angew. Chem. Int. (Eng. ed.) 19:344-362), including microscopic binding constants (Connors et al. (1984) J. Am. Chem.
- 25 Soc., 106:7607-7614). The crystalline complexes of drugs with cyclodextrins represent an encapsulation of drugs on the molecular level; molecules of drug are individually encapsulated in and separated from each other by cyclodextrin molecules; these complexes have established
- 30 importance in pharmaceuticals (see, e.g., Frank (1975) J. Pharm. Sci., 64:1585-1604). Recently, amorphous complexes of drugs with derivative of cyclodextrins were prepared and studied (see, e.g., Fenyvesi et al., (1984) Chem. Pharm. Bull., 32:670-677; Pitha (1984) Inclusion
- 35 Phenomena, 2:477-485; Kaji et al. (1985) Int. J. Pharm., 24:79-89; Pitha et al. (1985) J. Pharm. Sci., 74:987-991;

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and Uekama (1985) Pharm. Int. March 61-65). These complexes seem to combine the advantages that either the cyclodextrin complexation or the conversion of the drug to an amorphous state may exert on drug absorptions. One of the cyclodextrin derivatives used for the preparation of such amorphous complexes was hydroxypropyl-beta-cyclodextrin, the condensation product of cyclodextrin with propylene oxide. The preparation of hydroxypropyl-beta-cyclodextrin had been mentioned in U.S. patents (3,459,731 and 3,453,259), and that compound was investigated in previous studies (Pitha (1984) Inclusion Phenomena, 2:477-485; Pitha et al. (1985) J. Pharm. Sci., 74:987-991). The effects of hydroxypropyl-beta-cyclodextrin upon solubilities of various drugs has been studied (Pitha et al. (1986) Int. J. Pharm., 29:73-82).

Amphotericin B has previously been solubilized and stabilized by inclusion in gamma-cyclodextrin (see, e.g., Rajagopalan et al. (1986) Int. J. Pharm., 29:161-168; and Vikmon et al. (1985) J. Antibiotics, 38:1822-1824).

The present invention involves the creation and use of a new antifungal agent, namely amphotericin B included in hydroxypropyl-gamma-cyclodextrin.

25

The present invention concerns a composition of matter comprising antifungal polyene macrolide and hydroxyalkylated cyclodextrin. The hydroxyalkyl is preferably hydroxypropyl, more preferably, 2-hydroxypropyl. Hydroxyalkyl functions usable in the practice of the present invention may also include hydroxyethyl, hydroxyisobutyl, and 2, 11, 12-trihydroxy-4, 9-dioxadodecyl. The hydroxyalkyl cyclodextrin of the present invention may have six to eight glucose units, most preferably seven (beta cyclodextrin) or eight (gamma cyclodextrin), bound in 1-4 glucosidic linkages, each

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glucose unit having a hydroxyalkyl radical in ether linkage. The more preferably hydroxyalkyl cyclodextrins have seven cyclized glucosic units (beta cyclodextrin) or eight cyclized glucosic units (gamma cyclodextrin).

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The usual hydroxyalkyl cyclodextrin derivatives have glucose units each having a hydroxyalkyl radical in ether linkage. The hydroxyalkyl radical is most preferably bound in ether linkage to carbon 2, 3 or 6 of the glucose unit or to the carbon-2 of another hydroxypropyl radical. The hydroxyalkyl cyclodextrins involved in the present invention have glucose units bound in 1-4 glucosidic linkages, each glucose unit having a hydroxyalkyl radical in ether linkage. The ether linkage connecting the hydroxyalkyl substituent is to a carbon 2,3 or 6 of the glucose unit.

The antifungal polyene macrolide preferably used in the composition of matter and method of the present invention is most preferably amphotericin B, nystatin, natamycin, mepartricin, candicidin or lagosin.

The present invention further involves a method for treating a mammal with a disseminated fungal infection such as a yeast infection. Particularly infective yeasts which may be treated include Candida albicans, Candida tropicalis, Candida parapsilosis and Torulopsis glabrata infection. This method of treatment most preferably comprises parenteral and/or oral administration of the composition of matter described above comprising a cyclodextrin and an antifungal polyene macrolide to a mammal with a fungal infection. In a more preferred embodiment, the present invention involves administering to the mammal a fungicidally effective amount of a composition of matter comprising antifungal polyene macrolide and hydroxyalkyl cyclodextrin. The preferred

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fungicidally effective amount useful for therapy according to the present invention contains between about 0.4 mg antifungal polyene macrolide per kg body weight and about 4.0 mg antifungal polyene macrolide per kg body weight.

5

The present invention further involves a composition of matter comprising amphotericin B and hydroxyalkyl-gamma-cyclodextrin. The hydroxyalkyl-gamma-cyclodextrin is preferably hydroxypropyl-gamma-cyclodextrin, most preferably 2-hydroxypropyl-gamma-cyclodextrin. The cyclodextrin utilized in the practice of the present invention comprises rings of eight glucose units bound in 1-4 glucosidic linkages, each glucose unit having a hydroxyalkyl radical in ether linkage. The composition of matter of the present invention preferably comprises a cyclodextrin ring of eight glucose units bound in 1-4 glucosidic linkages, substantially each glucose unit having a 2-hydroxypropyl radical in ether linkage.

20 The present invention also involves methods for the treatment of an animal with fungal infection or the prophylaxis of fungal infection. This method may comprise administering to the animal a fungicidally effective amount of a composition of matter as described above comprising amphotericin B and hydroxyalkyl-gamma-cyclodextrin. The administration is preferably parenteral, particularly for disseminated fungal infection, but could be internal or topical, depending upon the particular site of fungal infection and desires of the treating physician. Topical treatment may include administration of an aerosolized amphotericin B-cyclodextrin complex. The disseminated fungal infection most preferably treated is a yeast infection such as a Candida albicans, Candida tropicalis, Candida parapsiloses or Torulopsis glabrata infection. The fungicidally effective amount useful for treatment of disseminated

fungus infection contains between about 0.4 mg amphotericin B per kg body weight and about 4.0 mg amphotericin B per kg body weight.

- 5 Figure 1 schematically shows the chemical structure of gamma-cyclodextrin (where R is H) and hydroxypropyl-gamma-cyclodextrin (where R is $-\text{CH}_2-\text{CHOH}-\text{CH}_3$).

- 10 Figure 2 graphically shows the relationship of amphotericin B solubility to the addition of gamma - cyclodextrin and hydroxypropyl-gamma-cyclodextrin.

- 15 Figure 3 shows the relationship of natamycin aqueous solubility to the presence of 200 mg cyclodextrin complex.

Hydroxyalkyl cyclodextrin may be synthesized according to the methodologies outlined in the following manuscripts.

- 20 1. Pitha J. and J. Pitha; Amorphous water soluble derivatives of cyclodextrins: Nontoxic dissolution enhancing excipients. Journal of Pharmaceutical Sciences. 74(9), 987-990, 1985.
- 25 2. Pitha J., J. Milecki, H. Fales, L. Pannel, K. Uekama; Hydroxypropyl-beta-cyclodextrin; Preparation and characterization; effect on solubility of drugs. International Journal of Pharmaceutics. 29, 73-82, 1986.

- 30 When amphotericin B is included in hydroxyalkyl-gamma-cyclodextrin or gamma-cyclodextrin (g-CD) the solubility of amphotericin B in an aqueous solution is drastically increased. Likewise, the solubility in an aqueous solution of amphotericin B is increased more
- 35 greatly by hydroxyalkyl-gamma-cyclodextrin than by g-CD. This phenomenon was demonstrated by including amphotericin

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B with γ -CD or the prototypical hydroxyalkyl- γ -cyclodextrin, 2-hydroxypropyl- γ -cyclodextrin (HP- γ -CD) in the following manner.

5 One mg of amphotericin B was added to each of a series of 1 ml aliquots of an aqueous solution containing increasing concentrations of HP-CD. In another set of similar experiments, γ -CD was substituted for HP- γ -CD. Each suspension was stirred at 37°C for three
10 hours and filtered. The filtrate was diluted with 50% ethanol/dimethylsulfoxide and the concentration of amphotericin B analyzed using a Beckman 25 UV spectrophotometer. The absorption wavelength used to
15 quantitate amphotericin B was 408 nm. The results as shown in Figure 3 indicated that HP- γ -CD resulted in almost 300% improvement in the aqueous solubility of amphotericin B compared to the solubility of amphotericin B conferred by γ -CD.

20 The toxicity of free amphotericin B and amphotericin B complexed with HP- γ -CD toward certain mammalian cells was determined. Fresh, washed human red blood cells (RBC) were added, at a final concentration of 2%, to tubes containing free amphotericin B (originally made in
25 dimethyl formamide at a level of 4%), or HP- γ -CD-amphotericin B complex. The samples were incubated for 45 minutes at 37°C, centrifuged at 10,000 x g for 20 minutes and the released hemoglobin in the supernatant determined by its light absorbance at 500 nm. Release of hemoglobin
30 by hypotonic lysis of the same number of cells in H₂O was also measured and was considered to represent 100% lysis. Table 1 shows the results of this experiment.

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TABLE 1
IN VITRO TOXICITY OF
AMPHOTERICIN B-HYDROXYPROPYL CYCLODEXTRINS COMPLEX

5			
	Drug tested	Concentration (ug/ml)	Human red blood cell toxicity % hemolysis
10			
	Amphotericin B	50	100
		100	100
15	Amphotericin B-	50	5
	HP-g-CD complex	100	1

20 It is clear from the results obtained that complexation with HP-g-CD led to a significant reduction of RBC toxicity of amphotericin B (from 100% to less than or equal to 5%).

25 The in vitro antifungal activity of free amphotericin B, amphotericin B complexed with g-CD and amphotericin B complexed with HP-g-CD was determined with various yeasts.

30 The yeasts to be tested were suspended in sterile saline to a "MacFarland #1" standard (approximately 85% light transmission at 540 nm). Drug solutions were prepared in buffered yeast nitrogen base broth at an initial concentration of 100 ug/ml. Twofold serial dilutions were made from the first well in the microtiter plate used through well 11. No drug was added to well 12
35 (negative control) and all wells of the microtiter plate

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were inoculated. The plate was incubated at 30°C for 24 hours. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented visible growth. Four yeast strains were tested; Candida
5 albicans, C. tropicalis, C. parapsilosis, and Torulopsis
glabrata. The results of this experiment are shown in Table 2.

TABLE 2

IN VITRO ANTIFUNGAL ACTIVITY OF FREE AMPHOTERICIN B AND AMPHOTERICIN B
ENCAPSULATED IN γ -CD AND HP- γ -CD AGAINST FOUR DIFFERENT FUNGAL SPECIES

MINIMAL INHIBITORY CONCENTRATION (MIC) IN $\mu\text{g/ml}$

Fungal strain	Amphotericin B in DMFA*	Amphotericin B in γ -CD+	Amphotericin B in HP- γ -CD++
<i>Candida albicans</i>	0.78	0.78	0.78
<i>Candida tropicalis</i>	0.78	0.78	0.78
<i>Candida parapsilosis</i>	0.78	0.78	0.78
<i>Torulopsis glabrata</i>	0.78	0.78	0.78
* Dimethylformamide			
+ γ -cyclodextrin			
++ 2-Hydroxypropyl- γ -cyclodextrin			

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MICs were identical (0.78 ug/ml) demonstrating that there was no loss of antifungal activity when amphotericin B is complexed with g-CD or HP-g-CD.

5 An important advantage of the amphotericin B-hydroxypropyl-g-cyclodextrin complex over amphotericin B-g-cyclodextrin complex was illustrated by a 300% improvement in aqueous solubility, which should translate into higher bioavailability of amphotericin B, decrease in
10 any potential toxicity from cyclodextrins, and significant financial savings. The simplicity of the preparation and the abolition of the in vitro toxicity of amphotericin B will make of this complex a suitable alternative to
15 amphotericin B for use in antifungal treatment and prophylaxis. Furthermore, this will allow the development of tablet and cream forms of the drug.

 The in vivo antifungal activity of free amphotericin B, amphotericin B complexed with g-CD and amphotericin B
20 complexed with HP-g-CD was determined with animals having disseminated infections by fungi such as various yeasts. The experimental scheme for this testing was as follows: A strain of *Candida albicans* (C.A. 366) isolated from a patient with disseminated candidiasis was chosen to infect
25 Halstoner mice 6-8 weeks years old, 18-20 gms. weight. The inoculum was cultivated for 18 hours on Sabouraud dextar agar, and diluted in .9% sodium chloride. An inoculum of 1×10^6 colony forming units/ml was prepared using 54% transmittance at 540 nm with a Beckman
30 spectrophotometer. This inoculum concentration was double checked by a hemocytometer reading. The animals were injected via the tail vein with this inoculum and animal survival was then assessed daily until death or 35 days follow-up, whichever came first. This model has been
35 repeatedly shown to be reproducible in that by day 2 all animals usually have disseminated candida infection as

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documented by both culture and histopathology. Control mice, i.e., untreated, would die within ten days. Drugs utilized were as follows: amphotericin B (fungizone)-0.8 mg/kg (maximum dose); Hydroxypropyl gamma cyclodextrin - amphotericin B complex as used at the same dose for comparison. Only one dose of either fungizone or hydroxypropyl gamma cyclodextrin - amphotericin B was used. Follow up data reveals that while control animals all died within 11 days, treated mice survived for a median of 32 days. There was no significant difference in the survival time between fungizone treated mice and those treated with the cyclodextrin complex of amphotericin B.

The hydroxypropyl gamma cyclodextrin - amphotericin B complex remained in solution in a stable state for more than 9 months after preparation, while the gamma cyclodextrin-amphotericin B became cloudy around 24-48 hours after preparation. Therefore, not only was the water solubility of amphotericin B improved by using hydroxypropyl gamma-cyclodextrin instead of gamma-cyclodextrin, but also the stability of the complex was significantly improved.

The encapsulation of insoluble lipophilic polyene antibiotics other than amphotericin B with hydroxypropyl preparations of cyclodextrin was also attempted. The first compound utilized was natamycin (also known as pimaracin). It is a tetraene that has a good in vitro activity against most yeast, although inferior to amphotericin B. However, natamycin has a significantly superior activity on molds, in particular aspergillus, as compared to amphotericin B. Therefore, it was of interest to formulate a new drug that has potent anti-aspergillus activity.

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When natamycin was included in a hydroxypropyl gamma cyclodextrin, gamma cyclodextrin or hydroxypropyl beta cyclodextrin complex, the solubility in a water solution of natamycin was increased more greatly by hydroxy alkyl
5 gamma cyclodextrin or hydroxy alkyl beta cyclodextrin then by beta or gamma cyclodextrin. This phenomenon was demonstrated by including natamycin with gamma cyclodextrin, hydroxypropyl gamma cyclodextrin, and hydroxypropyl beta cyclodextrin in the following manner.

10

One milligram of natamycin was added to each of a series of 1 ml aliquots of water solution containing increasing concentrations of the beta cyclodextrins at 37°, held for 3 hours and filtered. The filtrate was
15 diluted with 50% ethanol-dimethyl sulfoxide and the concentration of natamycin was analyzed using a Beckman 25UB spectrophotometer. The absorption wave-length used to quantitate natamycin was 330 nanometer. The results are shown in Fig. 3. The complexes formed by
20 hydroxypropyl beta or gamma cyclodextrin and natamycin remained in solution for more than 9 months after preparation. Solutions of beta or gamma cyclodextrin complexes with natamycin became cloudy and showed subsequent precipitation of the drug within 48 hours of
25 preparation. The toxicity of natamycin complexed with hydroxypropyl gamma-or hydroxypropyl beta-cyclodextrin toward certain mammalian cells was determined. Fresh human red blood cells (RBC) were added, at the final concentration of 20% to tubes containing natamycin (made
30 in dimethylformamide) or hydroxypropyl gamma CD-natamycin complex or hydroxypropyl beta CD-natamycin complex. The samples were incubated for 45 minutes at 37°C, centrifuged at 10,000g for 20 minutes and the hemoglobin content of the supernatant determined by its light absorbance. The
35 release of hemoglobin by distilled water was also measured and considered to represent 100% lysis. Table 3 shows the

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results of this experiment. It is clear from the results obtained that complexation with hydroxypropyl gamma-or beta-cyclodextrin led to a significant reduction of natamycin-induced RBC toxicity (from 75% to less than or equal to 0.2%).

The in vitro antifungal activity of free natamycin, natamycin complexed with hydroxypropyl gamma or hydroxypropyl beta-cyclodextrin was determined with various yeasts. The yeasts to be tested was suspended in sterile saline to a McFarland #1 standard (approximately 5% light transmittance at 540 nm). Drug solutions were prepared in buffered yeast nitrogen broth at initial concentrations of 100 ug/ml. Two-fold serial dilutions were made from the first well in the microtiter plate through well 11. No drug was added to well 12 (negative control) and all wells of the microtiter plate were inoculated with 100 microliters of the yeast solution. The plate was incubated at 30°C for 24 hours. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented visible growth. Four yeast strains were tested: Candida albicans, Candida tropicalis, Candida parapsilosis and Torulopsis glabrata. The results of the experiment are shown in Table 3. MIC's were identical (1.56 - 3.12 ug/ml) demonstrating that there was not loss of antifungal activity when natamycin is complexed with hydroxypropyl gamma or hydroxypropyl beta cyclodextrin. An important advantage of the natamycin hydroxypropyl cyclodextrin complex over natamycin cyclodextrin complex was illustrated by a 200-300% improvement in water solubility. In addition, hydroxypropyl cyclodextrin maintained natamycin in solution with water up to a period of 9 months while cyclodextrin without the hydroxypropyl component resulted in a turbid solution with precipitation of natamycin

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within 24 hours. The simplicity of the preparation and the abolition of the in vitro toxicity of natamycin will make of this complex a suitable alternative to amphotericin B for the use of antifungal treatment and
5 prophylaxis. Furthermore, this will allow the development of tablets, sprays, suppositories, and cream forms of the drug.

TABLE 3

IN VITRO ANTIFUNGAL ACTIVITY OF FREE NATAMYCIN AND NATAMYCIN
ENCAPSULATED IN HP8-CD AND HP-B-CD AGAINST FOUR FUNGAL SPECIES

MIMINAL INHIBITORY CONCENTRATION (MIC) IN ug/ml

Fungal Strain	Natamycin	
	in DMFA	HP8-CD
Natamycin		HP-B-CD
Candida albicans	3.1	3.1
Candida tropicalis	3.1	1.56
Candida parapsilosis	3.1	3.1
Torulopsis glabrata	3.1	1.56

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Acute and chronic toxicity of natamycin encapsulated in hydroxypropyl beta or gamma cyclodextrin was tested in Halstoner mice (6-8 weeks old, 18-20 gm. weight). Mice were injected with increasing doses of natamycin. Doses of up to 450 ug/mouse of natamycin in 20% methanol were injected into animals without death. However, because of solubility problems of natamycin with methanol, higher doses could not be injected. Injection of a 20% methanol control solution resulted in no deaths. Hydroxypropyl beta cyclodextrin natamycin complex injected at increasing doses from 500 to 2000 ug/animal (200/ug increments/dose) resulted in no death, even at the dose of 2000 ug/mouse. Similar results were obtained with hydroxypropyl gamma cyclodextrin in which case doses of up to 2800 ug/mouse failed to result in any actual toxicity. In addition, animals were bled to obtain values for hematology and chemistry testing. The mean hemoglobin for 4 animals injected with hydroxypropyl beta Cd-natamycin was 16.4 very similar to a value of 16.2 from controls. At the 800 ug/mouse dose of hydroxypropyl beta Cd-natamycin, the hemoglobin value was again 16.1, not very different from the control. At the 2500 ug/mouse dose of hydroxypropyl gamma natamycin, the hemoglobin value was 16.06, again not significantly different from control. Mean and median values for hemoglobin of all animals were very similar. The blood urea nitrogen for animals injected with 200 ug/mouse hydroxypropyl beta CD-natamycin, 800 ug/mouse hydroxypropyl beta CD-natamycin, or 2500 ug/mouse hydroxypropyl gamma-natamycin were 23.3, 26 and 26.75 respectively, not very different from the control mice having a hemoglobin value of 2.5. Similarly, liver enzymes, particularly SGPT in animals treated with 200 ug/mouse of hydroxypropyl beta CD-natamycin, 800 ug/mouse of hydroxypropyl beta Cd-natamycin, or 2500 ug/mouse of hydroxypropyl gamma Cd-natamycin again were very close and were respectively as follows: 50, 44, and 41 for a value

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of 40 for the control mice. Also, all pathology material which consisted of both kidneys, liver, spleen, lungs, and brain (4 animals in each group, i.e., hydroxypropyl beta Cd-natamycin 200 mg/mouse, hydroxypropyl beta Cd-natamycin 5 800 ug/mouse, and hydroxypropyl gamma Cd-natamycin 2500 mg/mouse and control) were all reviewed by a single pathologist. After careful review, it was felt that there was no evidence whatsoever of any organ damage that could be seen by the light microscope. In conclusion, there was 10 no apparent toxicity even after intravenous administration of very high doses of hydroxypropyl beta or hydroxypropyl gamma cyclodextrin natamycin complexes to Halstoner mice for up to 3 months after injection.

15 Additional in vitro results concerning the activity of natamycin HP beta CD against various fungi were obtained. The number of species and organisms tested comparing natamycin to the encapsulated formulation with cyclodextrin was increased. Fifty Cryptococcus 20 neoformans, 30 Candida Tropicalis, 30 Candida glabrata, 30 Trichosporon, 30 Candida parapsilosis, 50 Fusarium spp., and 50 Aspergillus spp. strains were tested. The results with the tests showed that encapsulation of natamycin with hydroxylpropyl-beta or hydroxylpropyl-gamma-cyclodextrin 25 maintained the antifungal activity of the compound. In particular, the minimum inhibitory concentrations (MIC₉₀), i.e., the MIC inhibiting 90% of in vitro growth were as follows: One ug/ml for Cryptococcus neoformans, 2 ug/ml for Candida tropicalis, glabrata, and parapsilosis; 1 30 ug/ml for Trichosporon, 2 ug/ml for Fusarium spp., and 1 ug/ml for Aspergillus spp. Based on in vivo data, these MIC values were well below the non-lethal blood levels that could be achieved in dogs with natamycin encapsulated in cyclodextrin. Hence, there are clear advantages of 35 therapeutically using this natamycin-beta cyclodextrin complex. Blood levels of a drug much higher than the MIC

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are associated with excellent therapeutic responses. This is a unique finding which can make of this drug - cyclodextrin complex - a breakthrough in antifungal chemotherapy. This new complex should be a valuable
5 chemotherapeutic agent, particularly for the treatment of Cryptococcus neoformans meningitis in patients with AIDS.

The solubilization of natamycin with novel cyclodextrin derivatives was additionally studied. The
10 encapsulation of natamycin with beta-cyclodextrin was compared to encapsulation into two new CD derivatives 6-0-alpha-D-glycosyl beta-CD (G-beta-CD) and 6^A,6^D-di-O-alpha-D-glycosyl-beta-CD(2G-beta-CD)(Kyoko, K., Yasuyo, O., Toshiko, U.: Inclusion complexes of poorly water-
15 soluble drugs with glucosyl-cyclodextrins. Chem. Pharm. Bull. 1987, 35(8):3413-3418). One hundred milligram of each G-beta-CD, 2G-beta-CD, or beta-CD were mixed with 3 mg of natamycin, shaken for three hours at 37° - filtered and sterilized. The filtrate was diluted with 50%
20 ethanol-dimethyl sulfoxide and the concentration of natamycin analyzed using a Beckman 25UB spectrophotometer at an absorption wave-length of 330 nanometer. The results indicated that G-beta-CD and 2G-beta-CD-encapsulated natamycin products results in an almost 300%
25 improvement in the aqueous solubility of natamycin compared to the solubility of this drug conferred by beta-CD encapsulation (2500 ug/ml with G-beta-CD and 2G-beta-CD versus 845 ug/ml with beta-CD). In addition, solutions of beta-CD complexes with natamycin became
30 cloudy and showed subsequent precipitation of the drug within 48 hours of preparation while G-beta-CD and 2G-beta-CD complexes persisted in solution from more than 2 months. Hence, this demonstrates a unique and new finding which is significantly superior to the existing art with
35 beta-CD alone. The value of this finding was confirmed by additional tests shown as follows:

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The in vitro antifungal activity of free natamycin, natamycin complex with G-beta-CD or 2G-beta-CD was determined with various yeasts. The yeasts to be tested were suspended in sterile saline to a McFarland No. 1 standard (approximately 95% light transmittance at 540 nanometer). Drug solutions were prepared in buffered yeast nitrogen broth at initial concentrations of 100 ug/ml. Two-fold serial dilutions were made from the first well in the microtiter plate through well eleven. No drug was added to well 12 (negative control) and all wells of the microtiter plate were inoculated with 100 microliters of the yeast solution. The inoculated plate was incubated at 30°C for 24 hours. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented visible growth. Four strains were tested: Candida albicans, Candida tropicalis, Candida parapsilosis, and Torulopsis glabrata. MIC's were identical for these two complexes (1.56 - 3.12 ug/ml), demonstrating that there was no loss of antifungal activity when natamycin is complexed with G-beta-CD or 2G-beta-CD.

The in vitro toxicity of natamycin complexed with G-beta-CD or 2G-beta-CD towards mammalian cells was tested. Fresh human red blood cells were added, at the final concentration of 20% to tubes containing natamycin (made in demethylformamide), G-beta-CD or 2G-beta-CD cyclodextrin-natamycin complex. The samples were incubated for 45 minutes at 37°C, centrifuged at 10,000 xg for 20 minutes and the hemoglobin content of the supernatant determined by its light absorbance. The release of hemoglobin by distilled water was also measured and considered to represent 100% lysis. The results showed that complexation with G-beta-CD or 2G-beta-CD led to a significant reduction of natamycin - induced red

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blood cell toxicity (from 75% to less than or equal to 1%).

Acute in vivo toxicity of natamycin encapsulated in G-beta-CD or 2G-beta-CD was tested in CF1 outbred mice (6-2 weeks old, 24 gm weight) Mice were injected with increasing dose of natamycin. Doses of up to 450 ug/mouse of natamycin in 20% methanol were injected into animals without death. However, injection of G-beta-CD or 2G-beta-CD natamycin complexes at increasing doses from 500 to 2,000 ug/ml resulted in no death, even at the dose of 2,000 ug/mouse. For the first time, these data show that natamycin can be encapsulated in G-beta-CD and 2G-beta-CD with persistence of antifungal activity and significant reductions of in vitro and in vivo toxicities toward mammalian cells or mammals.

The in vivo toxicity and pharmacology in higher animals (dogs) were also determined. Dogs weighing 20 kg were injected with natamycin solubilized in HP-beta-CD. These injections were given as an intraveous bolus over a 10 seconds period. In the first set of experiments, four dogs were injected with a 2.5 mg/kg dose of natamycin encapsulated in HP-beta-CD. Serial determinations of natamycin plasma levels were obtained after 5, 15, 30, 60, 120, and 360 minutes. The peak level obtained after 15 minutes, was 10 ug/ml. This was not associated with any toxicity, particularly no acute toxicity. It is important to mention that amphotericin B, the polyene antifungal drug of choice currently used in humans, may result in sudden death from arrhythmias if dogs are injected over a 20 second bolus injection. Two dogs which were injected with a 0.6 mg/kg dose of amphotericin B as a 20 second bolus died instantly. The availability of these new data suggest that substitution of natamycin for amphotericin B may permit rapid administration of the antifungal

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compound, a significant advantage over the slow administration of amphotericin B which is supposed to be over a 4 to 6 hour period, by standard medical practice (Bodey, GP: Topical and systemic antifungal agents. Med. Clin. North Amer. 1988. 1988, 72(3):637-659). This new data also clearly shows that natamycin has significantly less acute toxicity than amphotericin B.

In a subsequent experiment, four dogs were treated with five daily consecutive doses of 2.5 mg/kg of natamycin. Again no acute or chronic toxicity (after three months) were observed in these dogs. Also, similar blood levels of 10 ug/ml were achieved. These blood levels are significantly superior to those achieved by amphotericin B (in the range of 0.5 to 1.5 ug/ml) and higher than MIC for most fungi (0.1 - 2 ug/ml), suggesting that these higher blood levels would be associated with an improvement in the response rate of disseminated fungal infections. Hence, the ability now exists to administer a potent polyene antifungal with significant decrease in toxicity over amphotericin beta (the "gold" standard), and over a much shorter period of time. In addition, the serum levels achieved with HP-beta-CD-natamycin are at least 10 fold higher than those obtained with amphotericin B, a finding suggesting a great potential for improvement in the response rate of fungal infections over amphotericin beta-CD. Given the poor tolerance and response of patients with AIDS and/or cancer to amphotericin beta, this discovery may represent a major breakthrough for the treatment of these two conditions (Bodey).

One of the major limitations of amphotericin beta in the treatment of fungal infection in humans is its lack of availability as an oral formulation. This is because of the very low absorption (bioavailability) from the

-25-

gastrointestinal tract. The reason for this poor absorption appears to be a poor dissolution rate. Efficient dissolution is known to be essential for absorption of drug through the gastrointestinal tract

5 (Frijlink, H.W., Schooner, A.J.M., Lerk, C.F.: The effects of cyclodextrins on drug absorption. I. In vitro observations. Int. J. Pharm. 1989, 49:91-102). The bioavailability of drugs from their solid state form depends primarily on their dissolution properties; fast

10 dissolution and the ability to form a concentrated solution usually result in good absorption of the drug. The dissolution characteristic of a compound is mainly a function of structure, but to a lesser degree can be changed by manipulation of the solid state of the drug.

15 Solvents of drugs differ in rates of dissolution and insolubility; the proper choice of the crystalline state does make it dissolution. A drug may also be converted from the crystalline state into an amorphous state which dissolves better. Unfortunately, the amorphous state is

20 metastable. The present invention concerns the complexation of polyene antibiotics with cyclodextrin. Cyclodextrin has been used extensively as a complexation agent, and has been associated with a significant improvement in the bioavailability of drugs. Hence,

25 amphotericin B and other polyenes were always considered not bioavailable. With the present invention, these drugs can now be give orally. This can result in simplification of antifungal chemotherapy and major savings in health costs. In times of spiralling medical costs and budget

30 restraints, it becomes essential to lower the cost of treating such deadly diseases as AIDS and cancer. It should be pointed out that this economical aspect of this invention is indeed very important as patients with AIDS and fungal infections may remain on antifungal therapy for

35 extended periods. With amphotericin B, this would be very costly as patients need to receive the drug intravenously,

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usually in the hospital setting. The antifungal complexes of the present invention should be an additional weapon in the armamentarium against raising medical costs, and should constitute a solid basis for the patentability of our invention.

One of the most serious problems affecting the U.S. agriculture is the increasing number of infection by molds, particularly Aspergillus flavus and Fusarium spp. causing major losses in crops, and potential cancer threats to humans ingesting contaminated products (A scare in the corn belt: Does a carcinogen threaten America's No. 1 crop? Newsweek; March 6, 1989, p. 70; and Spreading poison: Fungus in corn crop, a potent carcinogen, invades food supplies. The Wall Street Journal. Thursday, February 23, 1989). In an effort to determine whether the complexes of the present invention would be helpful in the control of these widespread infections, the following experiments were conducted. Potatoes were divided into slices and inoculated with a solution containing 1×10^3 colony forming units (CFU) of Fusarium solani. These potatoes were divided in two groups: one used as control, while the second one was treated twice daily for three consecutive days with the solution of HP-beta-CD (50 ug/ml) natamycin. Growth on potato slices was followed for 10 days, at which time potato slices, if showing no obvious growth, were subcultured onto Sabouraud dextrose agar to make sure that there was no persistence of fungus. The results of this experiment showed a total inhibition of growth of Fusarium solani on the potatoes that were treated twice daily with natamycin (zero CFU) as compared to the excessive growth all over the potatoes surface in the group that was for control (too many CFU's to count).

Again this is a new finding which substantiates the value of this complex for the control of contaminated

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crops and may be an important factor for the control of mold infections affecting agriculture and for which there is no current effective treatment (Newsweek, March 6, 1989, p. 70; and The Wall Street Journal, Thursday, February 23, 1989).

The following results relate to solubilization of different polyenes antibiotics with hydroxypropyl gamma cyclodextrin. Adequate concentration of various polyene antibiotics were obtained by adding 5-10 mg of the polyene together 200 mg of hydroxypropyl gamma CD and 1 ml of saline. For example, 5 mg of candicidin were added to 100 mg of hydroxypropyl gamma CD and 1 ml of saline giving a concentration of 100 mg/ml. Six mg of lagosin with 175 mg of hydroxypropyl gamma CD and 1 ml of saline gave a concentration of 600 ug/ml of lagosin. Ten mg of mepartricin + 200 mg of hydroxypropyl gamma cyclodextrin + 1 ml of saline gave a concentration of 1900 ug/ml. Ten mg of nystatin + 200 mg of hydroxypropyl gamma cyclodextrin + 1 ml of saline gave 3500 ug/ml. Five mg of candicidin + 100 mg of hydroxypropyl gamma cyclodextrin + 1 ml of saline gave a concentration of 1300 ug/ml. Results of solubilization of these various potent antibiotics with hydroxypropyl gamma cyclodextrin were at least as good or even better than solubilization of amphotericin B with the same amount of hydroxypropyl gamma cyclodextrin. In vitro RBC toxicities of the above mentioned polyenes also showed a dramatic decrease in RBC toxicity ranging from 75 to 100% red blood cell hemolysis with the three polyenes down to 0.01-2 less than 5% when these polyenes were encapsulated with hydroxypropyl gamma cyclodextrin. Additionally, there was no loss of in vitro antifungal activity as previously tested with these polyenes were encapsulated with hydroxypropyl gamma cyclodextrin as compared to the three polyenes. In addition, in vivo activity of candicidin encapsulated in hydroxypropyl gamma

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cyclodextrin revealed that encapsulation with hydroxypropyl gamma cyclodextrin results in similar in vivo activity than candicidin solubilized in dimethylsulfoxide 20% solution against a model of C. albicans 336 disseminated fungal infection in CF1 Swiss mice. These results establish that there is significant increase in the solubility of various polyenes antifungal antibiotics when encapsulated with hydroxypropyl gamma cyclodextrin without any loss of antifungal activity either in vitro or in vivo but with a significant decrease of human red blood cell toxicity.

The cyclodextrin-antifungal polyene macrolide combinations of the present invention also may prove useful in the prophylaxis and/or treatment of disease caused by human immunodeficiency virus (HIV), formerly T lymphotropic retrovirus, (HTLV-III/LAV). As Gallo pointed out, HIV may be carried in vivo by monocytes and macrophages (see, e.g., p 51, Scientific American, January, 1987, pp 47-56). These cells types may thus serve as potentially infections and deadly HIV reservoirs.

Schaffner et al. (Biochem. Pharmacol., V 35, pp 4110-4113 (1986)) showed data indicating that the replication of HIV in the monocyte-related cell line H9 was inhibited by several antifungal polyene macrolides. These polyene macrolides included amphotericin B and amphotericin B methyl ester ascorbate.

The phagocytes of the blood--monocytes, macrophages and polymorphonuclear leukocytes--characteristically bind and ingest foreign substances, even prior to an immune response. These phagocytes also are among the first cells to take up circulating foreign complexes. It appears likely that parenteral administration to an animal of cyclodextrin complexes comprising a polyene macrolide

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should be useful to inhibit intracellular HIV proliferation. Although studies confirming this usefulness of the cyclodextrin-antifungal polyene macrolides complexes have not yet been confirmed, it may
5 be predicted that the complexes of the present invention should have a role in therapy and by prophylaxis of AIDS.

CLAIMS:

1. A composition of matter comprising antifungal polyene
5 macrolide and hydroxy alkyl cyclodextrin.
2. The composition of matter of claim 1 wherein the
hydroxyalkyl cyclodextrin is hydroxypropyl cyclodextrin.
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3. The composition of matter of claim 2 wherein the
hydroxypropyl cyclodextrin is hydroxypropyl gamma
cyclodextrin.
15
4. The composition of matter of claim 1 wherein the
alkyl comprises five or less carbon atoms.
20
5. The composition of matter of claim 1 or 2 wherein the
cyclodextrin comprises a ring of six to eight glucose
units bound in 1-4 glucosidic linkages, each glucose unit
having a hydroxyalkyl radical in ether linkage.
25
6. The composition of matter of claim 1 or 2 wherein the
cyclodextrin comprises a ring of eight glucose units bound
in 1-4 glucosidic linkages, each glucose unit having a
30 hydroxyalkyl radical in ether linkage.
7. The composition of matter of claim 1 or 2 wherein the
cyclodextrin comprises a ring of seven glucose units bound
35 in 1-4 glucosidic linkages, each glucose unit having a
hydroxyalkyl radical in ether linkage.

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8. The composition of matter of claim 1 or 2 wherein the cyclodextrin is gamma cyclodextrin.
- 5 9. The composition of matter of claim 1 or 2 wherein the cyclodextrin is beta cyclodextrin.
- 10 10. The composition of matter of claim 1 or 2 wherein the cyclodextrin is gamma cyclodextrin and comprises glucose units having the hydroxyalkyl radical in ether linkage.
- 15 11. The composition of matter of claim 1 or 2 wherein the cyclodextrin is beta cyclodextrin comprising glucose units having the hydroxyalkyl radical in ether linkage.
- 20 12. The composition of matter of claim 1 or 2 wherein the cyclodextrin comprises rings of seven glucose units bound in 1-4 glucosidic linkages, each glucose unit having the hydroxyalkyl radical in ether linkage.
- 25 13. The composition of matter of claim 1 or 2 wherein the antifungal polyene macrolide is amphotericin B, nystatin, natamycin, mepartricin, candicidin or lagosin.
- 30 14. The composition of matter of claim 2 wherein the hydroxyalkyl radical is bound in ether linkage to carbon 2, 3 or 6 of the glucose unit or to the carbon-2 of another hydroxypropyl radical.

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15. The composition of matter of claim 2 wherein cyclodextrin comprises rings of eight glucose units bound in 1-4 glucosidic linkages, each glucose unit having a 2-hydroxypropyl radical in ether linkage.
5
16. A method for treating disseminated fungal infection comprising parenteral administration of the composition of matter of claim 1 to a mammal with a disseminated fungal
10 infection.
17. A method for treating a mammal having a disseminated fungal infection, the method comprising:
15
- administering to the mammal a fungicidally effective amount of a composition of matter comprising antifungal polyene macrolide and hydroxyalkyl
20 cyclodextrin.
18. The method of claim 17 wherein the administering is parenteral.
25
19. The method of claim 17 wherein the hydroxyalkyl cyclodextrin is hydroxypropyl cyclodextrin.
30
20. The method of claim 19 wherein the hydroxypropyl cyclodextrin is 2-hydroxypropyl cyclodextrin.
21. The method of claim 17 wherein the 2-hydroxyalkyl cyclodextrin comprises a ring of six to eight glucose
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units bound in 1-4 glucosidic linkages, each glucose unit having a hydroxyalkyl radical in ether linkage.

5 22. the method of claim 21 wherein the ether linkage is to a carbon 2,3 or 6 of the glucose unit.

23. The method of claim 17 wherein the disseminated
10 fungal infection is a yeast infection.

24. The method of claim 17 wherein the disseminated fungal infection is a *Candida albicans*, *Candida*
15 *tropicalis*, *Candida parapsilosis*, *Torulopsis glabrata* or *Aspergillus* spp., or *Phycomycetes*, or *Fusarium* spp. infection.

20 25. The method of claim 17 wherein the fungicidally effective amount contains between about 0.4 mg antifungal polyene macrolide per kg body weight and about 4.0 mg antifungal polyene macrolide per kg body weight.

25

26. The method of claim 18 wherein the antifungal polyene macrolide is amphotericin B, nystatin, natamycin, mepartricin, candicidin or lagosin.

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27. A composition of matter comprising cyclodextrin and amphotericin B, nystatin, natamycin, mepartricin, candicidin or lagosin.

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28. The composition of matter of claim 27 wherein the cyclodextrin is beta cyclodextrin or gamma cyclodextrin.
- 5 29. The composition of matter of claim 27 wherein the cyclodextrin is hydroxyalkyl cyclodextrin.
- 10 30. The composition of matter of claim 27 wherein the cyclodextrin is hydroxypropyl cyclodextrin.
- 15 31. The composition of matter of claim 27 wherein the cyclodextrin is 2-hydroxypropyl cyclodextrin.
- 20 32. The composition of matter of claim 27 wherein the cyclodextrin is hydroxypropyl gamma cyclodextrin.
- 25 33. The composition of matter of claim 27 wherein the hydroxypropyl gamma cyclodextrin is 2-hydroxypropyl gamma cyclodextrin.
- 30 34. A method for treating an animal with disseminated fungal infection comprising parenteral administration to said animal of a composition of matter comprising cyclodextrin and an antifungal polyene macrolide selected from the group consisting of amphotericin B, nystatin, natamycin, mepartricin, candicidin and lagosin.
- 35 35. A method for treating an animal with disseminated fungal infection comprising parenteral administration to

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said animal of a composition of matter comprising cyclodextrin and amphotericin B.

- 5 36. A method for treating an animal with disseminated fungal infection comprising parenteral administration to said animal of a composition of matter comprising cyclodextrin and nystatin.

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37. A method for treating an animal with disseminated fungal infection comprising parenteral administration to said animal of a composition of matter comprising cyclodextrin and natamycin.

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38. A method for treating an animal with disseminated fungal infection comprising parenteral administration to said animal of a composition of matter comprising
20 cyclodextrin and mepartricin.

39. A method for treating an animal with disseminated fungal infection comprising parenteral administration to
25 said animal of a composition of matter comprising cyclodextrin and candicidin.

40. A method for treating an animal with disseminated
30 fungal infection comprising parenteral administration to said animal of a composition of matter comprising cyclodextrin and lagosin.

- 35 41. The method of claim 34 wherein the cyclodextrin is hydroxyalkyl cyclodextrin.

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42. The method of claim 34 wherein the cyclodextrin is hydroxypropyl cyclodextrin.
- 5 43. The method of claim 34 wherein the cyclodextrin is hydroxypropyl gamma cyclodextrin or hydroxypropyl beta cyclodextrin.
- 10 44. The method of claim 34 wherein the cyclodextrin is 2-hydroxypropyl-gamma-cyclodextrin.
- 15 45. The method of claim 34 wherein the disseminated fungal infection is a yeast infection.
46. The method of claim 34 wherein the disseminated fungal infection is a *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Torulopsis glabrata*, *Aspergillus*, *Phycomyces* or *Fusarium* spp. infection.
- 20 47. The method of claim 34 wherein the composition of matter is administered in a fungicidally effective amount and the fungicidally effective amount contains between about 0.4 mg antifungal polyene macrolide per kg body weight and about 4.0 mg antifungal polyene macrolide per kg body weight.
- 30 48. A composition of matter comprising a natamycin and glycosyl hydroxyalkyl beta cyclodextrin complex.
49. The composition of matter of claim 48 wherein the hydroxyalkyl beta cyclodextrin complex is hydroxypropyl beta cyclodextrin complex.
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50. The composition of matter of claim 48 wherein the glycoxyl beta cyclodextrin is 6-O-alpha-D-glycosyl-beta cyclodextrin or 6^A,6^D-di-O-alpha-D-glycosyl-beta cyclodextrin.

5

51. The composition of matter of claim 48 wherein the hydroxyalkyl comprises five or less carbon atoms.

52. A method for treating disseminated fungal infection comprising parenteral administration of the composition of matter of claim 49 to a mammal with a disseminated fungal infection.

53. A method for treating a mammal having a disseminated fungal infection, the method comprising:

administering to the mammal a fungicidally effective amount of a complex comprising natamycin and hydroxyalkyl or glycosyl beta cyclodextrin.

20

54. The method of claim 53 wherein the administering is parenteral.

55. The method of claim 53 wherein the glycosyl cyclodextrin is 6-O-alpha-D-glycosyl or 6^A,6^D-di-O-alpha-D-glycosyl-beta cyclodextrin.

25

56. The method of claim 54 wherein the hydroxyalkyl cyclodextrin is 2-hydroxypropyl cyclodextrin.

30

57. The method of claim 53 wherein the disseminated fungal infection is a yeast infection.

58. The method of claims 53 wherein the disseminated fungal infection is a *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*,

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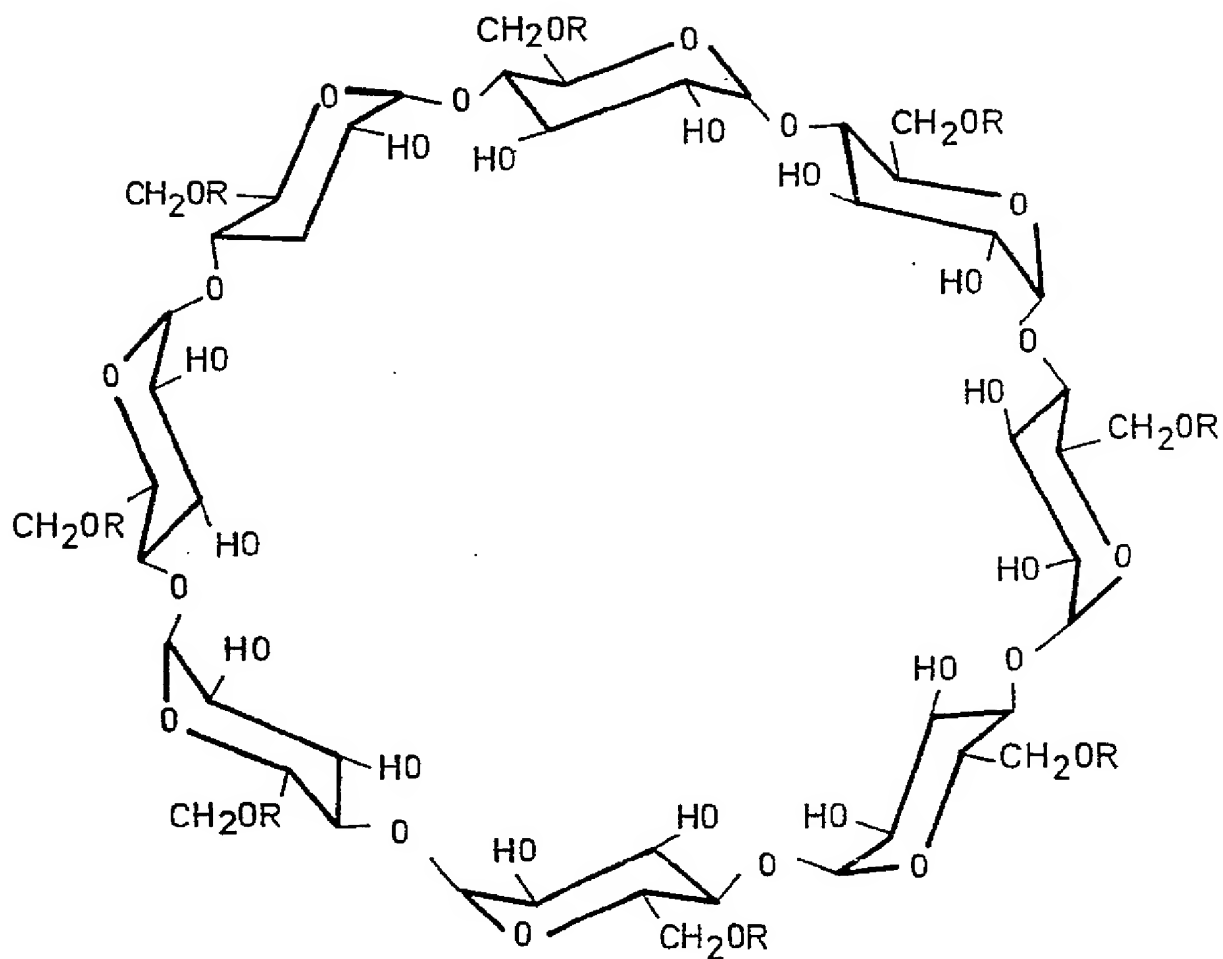
Torulopsis glabrata, *Aspergillus* spp., *Phycomycetes*, *Trichosporon*, *Cryptococcus neoformans* or *Fusarium* spp. infection.

- 5 59. The method of claim wherein the fungicidally effective amount contains between about 0.4 mg. natamycin per kg body weight and about 4.0 mg antifungal polyene macrolide per kg body weight.
- 10 60. A method for treating a plant or plant part with fungal infection comprising administration to said plant or plant part of a composition of matter comprising cyclodextrin or cyclodextrin derivative and an antifungal polyene macrolide selected from the group consisting of
15 amphotericin B, nystatin, natamycin, mepartricin, candicidin and lagosin.
- 20 61. A method for preventing or treating a plant or plant part with fungal infection comprising administration to said plant or plant part of a composition of matter comprising cyclodextrin or a cyclodextrin derivative and natamycin.
- 25 62. The method of claim 60 or 61 wherein the administration is topical.
63. The method of claim 60 or 61 wherein the cyclodextrin derivative is hydroxyalkyl cyclodextrin.
- 30 64. The method of claim 60 or 61 wherein the cyclodextrin derivative is cyclodextrin derivative.
- 35 65. The method of claim 64 wherein the beta cyclodextrin derivative is 6-O-alpha-D-glycosyl or 6^A,6^D-di-O-alpha-D.

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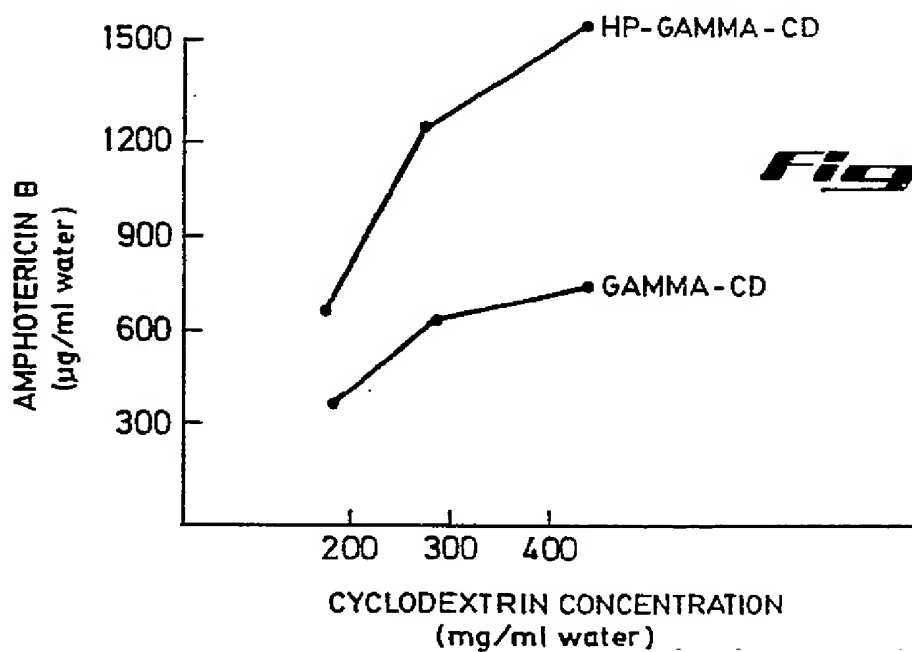
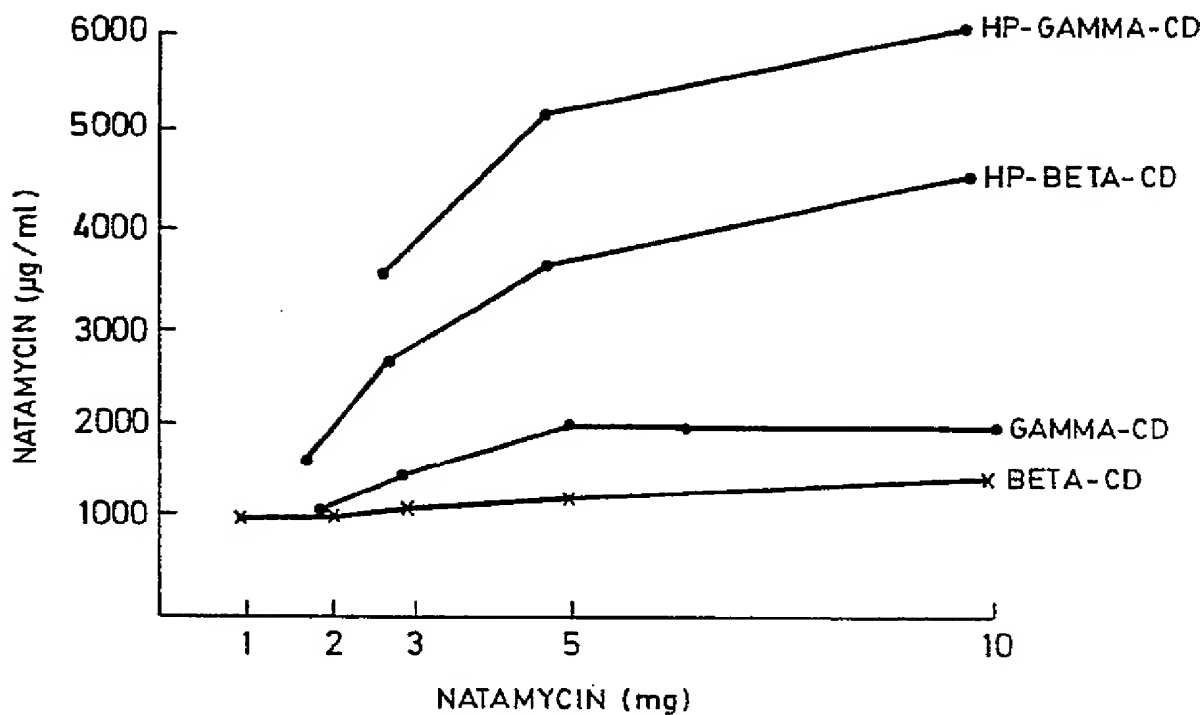
66. The method of claim 60 or 61 wherein the cyclodextrin is hydroxypropyl gamma cyclodextrin or hydroxypropyl beta cyclodextrin.
- 5 67. The method of claim 60 or 61 wherein the fungal infection is an Aspergillus or Fusarium infection.

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Fig. 1

SUBSTITUTE SHEET

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Fig. 3**Fig. 2**

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/01912

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : A 61 K 9/18, A 61 K 31/71											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="width: 75%; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">IPC⁴</td> <td style="border: 1px solid black; padding: 5px;">A 61 K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	A 61 K					
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ¹⁰</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">EP, A, 0147851 (CHINOIN) 10 July 1985, see claims; page 4, lines 20-24; page 14, lines 21-23; page 15, lines 11-17 <div style="text-align: center;">--</div></td> <td style="vertical-align: top; padding: 5px;">1-15, 27-33, 48-51</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">EP, A, 0197571 (JANSSEN) 15 October 1986, see claims 1-4, 8-10; page 5, lines 23-33; page 6, lines 1-6; page 8, lines 6-11 <div style="text-align: center;">-----</div></td> <td style="vertical-align: top; padding: 5px;">1-15, 27-33, 48-51</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	EP, A, 0147851 (CHINOIN) 10 July 1985, see claims; page 4, lines 20-24; page 14, lines 21-23; page 15, lines 11-17 <div style="text-align: center;">--</div>	1-15, 27-33, 48-51	Y	EP, A, 0197571 (JANSSEN) 15 October 1986, see claims 1-4, 8-10; page 5, lines 23-33; page 6, lines 1-6; page 8, lines 6-11 <div style="text-align: center;">-----</div>	1-15, 27-33, 48-51
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Y	EP, A, 0147851 (CHINOIN) 10 July 1985, see claims; page 4, lines 20-24; page 14, lines 21-23; page 15, lines 11-17 <div style="text-align: center;">--</div>	1-15, 27-33, 48-51									
Y	EP, A, 0197571 (JANSSEN) 15 October 1986, see claims 1-4, 8-10; page 5, lines 23-33; page 6, lines 1-6; page 8, lines 6-11 <div style="text-align: center;">-----</div>	1-15, 27-33, 48-51									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁴ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Δ" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 28th July 1989 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 1 8 AUG 1989 </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer M. VAN MOL </td> </tr> </table>			Date of the Actual Completion of the International Search 28th July 1989	Date of Mailing of this International Search Report 1 8 AUG 1989	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer M. VAN MOL					
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International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer M. VAN MOL										

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers * , because they relate to subject matter not required to be searched by this Authority, namely:

* 16-26, 34-47, 52-67

See PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers , because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING :

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8901912
SA 28821

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/08/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0147851	10-07-85	JP-A- 60158188	19-08-85
EP-A- 0197571	15-10-86	JP-A- 61275301	05-12-86
		US-A- 4764604	16-08-88

EP-A- 0147851

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82